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der Vetsuisse-Fakultät Universität Zürich

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Arbeit unter wissenschaftlicher Betreuung von
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Development of a flow cytometric assay to assess the bacterial count in boar semen

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

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2019

Inhalt

1. Abstract

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Etablierung einer durchflusszytometrischen Methode zur Bestimmung der Keimzahl in Ebersperma

Ziel der Studie war es, eine durchflusszytometrische Methode zur Bestimmung der Keimzahl in Ebersperma zu entwickeln.

Insgesamt wurden 224 frische Ejakulate von KB-Ebern analysiert. Die Gesamtzahl der lebenden Bakterien wurde nach Färbung mit SYBR Green I und Propidiumjodid (PI) mittels Durchflusszytometrie bestimmt. Im ersten Teil der Studie wurden 111 Spermaproben mit definierten Keimzahlen von Reinkulturen von häufig in Eberejakulaten vorkommenden Bakterienarten versetzt und anschliessend durchflusszytometrisch analysiert. Im zweiten Teil der Studie wurden 113 Spermaproben am Tag der Gewinnung sowohl mittels Durchflusszytometrie als auch mittels Most Probable Number (MPN) Methode als bakteriologische Standardmethode untersucht.

Im ersten Teil der Studie zeigte sich eine starke Korrelation zwischen gemessenen und erwarteten Keimzahlen ($r = 0,96$; $P < 0,001$), während im zweiten Teil die Werte der durchflusszytometrischen Methode und die der MPN-Methode moderat korrelierten ($r = 0,28$; $P < 0,01$; Median MPN: 24.000 ± MAD 21.600 Bakterien/ml; Median Durchflusszytometrie: 24.426 ± MAD 15.610 Bakterien/ml).

Die Durchflusszytometrie bietet somit eine zeitsparende Alternative zur klassischen mikrobiologischen Technik um kontaminierte Eberejakulate zu erkennen. Das entwickelte Protokoll ermöglicht mit überschaubarem Aufwand die Zahl der lebenden Bakterien in frischen Ejakulaten zu bestimmen, sodass die Möglichkeit eines Einsatzes während der Produktion in KB-Stationen gegeben ist.

Schlüsselwörter: Durchflusszytometrie, Bakterien, Eber, Sperma

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Development of a flow cytometric assay to assess the bacterial count in boar semen

The aim of the study was to develop a new flow cytometric assay for the determination of the bacterial count in commercially processed boar semen.

In total 224 fresh boar semen samples collected at an AI-station were analyzed. The number of total viable counts (TVC) was determined by using flow cytometry after staining with SYBR Green I and Propidium Iodide (PI). In the first part of the study 111 fresh boar semen samples were spiked with pure cultures of defined numbers of bacteria commonly detected in boar ejaculates and analyzed by flow cytometry. In the second part, 113 fresh semen samples were assessed on the day of collection through flow cytometry and the Most Probable Number (MPN) method, as the standard bacteriological method.

The first part of the study showed a strong correlation between the detected and expected numbers ($r = 0.96$; $P < 0.001$), while in the second part of the study the TVC determined by flow cytometry and by the MPN method correlated only moderately ($r = 0.28$; $P < 0.01$; median MPN: 24,000 ± MAD 21,600 bacteria/mL; median flow cytometry: 24,426 ± MAD 15,610 bacteria/mL).

In summary flow cytometry is a fast alternative to the classical culture technique to determine highly contaminated boar ejaculates. The developed flow cytometric protocol enables one to enumerate the viable bacteria within fresh boar ejaculates without requiring numerous treatment steps, and thus offering the possibility of an on-line use in AI-centers.

Keywords: Flow cytometry, bacteria, semen, boar

2. Introduction:

In the modern pig industry, artificial insemination (AI) plays a key role for successful and cost-efficient animal breeding and production. The application of AI in swine industry is constantly expanding, not only in the industrialized countries but also in emerging economies like East Asia and South America [1]. With one boar being able to serve about 2000 sows per year, AI is by far more efficient than natural breeding. AI also simplified the spread and exchange of genetic potential even beyond national boundaries [1,2]. However, this comes along with an increased risk of spreading diseases via preserved semen thus necessitating a high standard of sanitary control [3].

Due to the process of collection, boar semen usually contains bacteria [4]. Commercially processed boar semen is diluted and stored in liquid phase at 17 °C up to 6 days after collection and dilution. Bacterial growth is in fact reduced, but still not as suppressed as by storage in liquid nitrogen. For this reason antibiotics are commonly included in boar semen extenders [5]. In order to prevent spreading of diseases, national and international regulations stipulate that antibiotics have to be part of the extender (OIE, 2016; EU Directive 90/429/EEC).

Starting off with the collection of the semen, the contamination of the native ejaculate should be as low as possible to ensure a good basis for further processing steps [6]. Bacteriospermia can lead to reduced sperm longevity and also be a source for disease spreading [7]. Depending on the type of bacteria species and the contamination level of the ejaculate, motility and viability of sperm decreases and the rate of sperm agglutination increases [3,7].

Several studies have shown that contamination during the production process in the lab is a common problem which can even lead to antimicrobial resistant bacteria populations in the final semen dose [8]. With emerging resistances against the antibiotics commonly used in commercial extenders, it is crucial to ensure a hygienic production process.

As part of the quality control in boar AI stations, the final semen doses are regularly checked in terms of total viable counts (TVC) [4,9,10]. The samples are cultured on blood agar at 37 °C for 24-48hours (h). Due to the lack of a faster and less labour intensive method, checking each

ejaculate is impractical and so far only random samples are tested to monitor the levels of production hygiene [11].

Furthermore, most semen doses might already be sold by the time the results of bacteriological tests are available, a problem that could only be solved through the application of faster methods for the bacteriological examination of semen samples.

In contrast to the standard bacteriological methods, flow cytometry is a technique suitable to analyze a large amount of cells in a short time. Due to technical improvements, the sensitivity of flow cytometers is constantly increasing, so that nowadays even small cells like bacteria can be reliably detected.

The large variety of available fluorescent dyes facilitates the quantitative assessment of bacterial populations while it simultaneously enables the analysis of different properties and physiological stages of bacterial cells [12,13].

There are various flow cytometric protocols describing the enumeration of bacteria in urine [14,15,16], marine water [17] or drinking water [18,19]. For complex biological materials like blood [20], milk [21,22,23], plants [24] and even soil [25] relevant protocols are based on segregating bacteria from the ambient media by methods such as lysis or centrifugation; however these steps often require additional time and specialized equipment.

Thus far, there is no flow cytometric protocol for the determination of TVC in fresh ejaculates in a fast and easy way so that it would be feasible for routine testing. Therefore, this study aims to develop a protocol for the assessment of TVC in semen that could be routinely applicated in AI stations.

3. Material and methods:

3.1 Experimental design

The study consisted of two parts: a) the development of a protocol for the flow cytometric detection of bacterial populations in semen samples spiked with pre-defined numbers of pure

bacterial cultures, and b) the comparative assessment of TVC through a classical microbiological culture technique and the new flow cytometric protocol.

For the first part, pure cultures of seven different bacteria species commonly found in boar ejaculates were used as single species samples as well as a mixed sample, stained with SYBR Green I and PI and measured separately by flow cytometry. Afterwards semen and bacteria were combined, stained and measured as described above, in order to identify the region of interest for viable bacteria. To test whether the measurement is reliable at different concentrations of bacteria, dilution series were measured as well by performing five dilution steps on four ejaculates.

Subsequent, TVC was determined with flow cytometry in a total of 111 semen samples and the pure cultures of the seven bacteria species. Then semen and bacteria samples were mixed and measured again. The TVC of the spiked sample was then compared to the number calculated by summating the count of the unspiked semen and bacteria sample. All measurements were carried out in duplicates and each bacteria species was tested in at least 10 different ejaculates.

For the second part of the study, 113 raw semen samples were split up in two aliquots. The first one was left untreated while the second one was diluted to prevent agglutination. Both aliquots were kept at 17 °C until being processed on the same day. From the first aliquot serial were inoculated and enumerated using the Most Probable Number (MPN) method after 48h culturing at 37 °C. The second one was stained with SYBR Green I and PI and TVC were determined in duplicates by flow cytometry.

3.2 Collection of the semen samples

In total 224 fresh boar semen samples from a commercial AI boar stud (SUISAG, Sempach, Switzerland) were used. Samples were collected by the gloved-hand technique [26] and immediately processed. Boars of different age and breeds (Duroc, Pietrain, Premo®) were randomly chosen.

3.3 Semen samples for spiking

For the spiking experiments raw semen samples were diluted 1:10 in Tyrode solution (NaCl 100mM, KCl 3.1 mM, $CaCl_2$ 2.0 mM, $MgCl_2$ 0.4 mM, NaH_2PO_4 0.3 mM, $NaHCO_3$ 25 mM, Na-lactate 21.6 mM, Na-Pyruvate 1.0 mM, HEPES 10 mM, 0.5 mg/mL PVP, 0.5 mg/mL PVA; adjusted to pH 7.54 and an osmolarity of 320 mOsmol/kg and filtered through a 0.2µm pore size filter) right after collection to prevent agglutination of sperm cells. During transport to the lab and storage, they were kept at 17 °C in a temperature-controlled box until analyses were performed the following day.

3.4 Bacteria for spiking

For spiking, pure cultures of bacteria commonly found in fresh boar semen were used [3]: *Staphylococcus aureus, Streptococcus* spp*., Aeromonas* spp*., Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae* and *Escherichia coli.* The bacteria were isolated in the routine diagnostic laboratory from different swine samples. Species identification was done by MALDI-TOF MS (Bruker, Bremen, Germany). All bacteria were streaked on Columbia blood agar (Thermo Fisher Diagnostics AG, Pratteln, Switzerland) and a McFarland suspension of 1 (1 MCF = $3x10^8$cfu/mL) was prepared using NaCl 0.9%. Prior to staining the samples were diluted 1:10 resulting in a $3x10^7$ cfu/mL concentration.

For the dilution series, five different concentrated solutions were prepared, from 1 MCF down to a $4.5x10^6$ cfu/mL suspension.

3.5 Fluorescence staining

Stock solutions of SYBR Green I (10 000x in DMSO; SYBR® Green I nucleic acid gel stain, Molecular Probes supplied by LifeTechnologies, Eugene, Oregon, USA) and Propidium Iodide (10mg PI P 4170, Sigma-Aldrich Chemie GmbH, Steinheim, Germany in 5ml aqua bidest) were prepared and a Mastermix consisting of SYBR Green I and PI diluted 1:100 in sterile filtered water (0.2µm, Filtropur S Sarstedt, Nümbrecht, Germany) was set up. To ensure that there is no

contamination in the Mastermix, a sample of each lot was checked prior to use by determining the bacterial count via flow cytometry.

3.6 Sample preparation

Prior to the measurement semen samples were diluted 1:10 using the Mastermix, thoroughly mixed for 2 sec (Vortex RS-VA10, Phoenix Instrument, Garbsen, Germany) and incubated in the dark at 37 °C for 15 min.

Bacterial enumeration in commercial drinking water as Evian® (Evian, France) has been established by Hammes et al. [18]; thus, a sample of unfiltered Evian® water served as reference sample and was stained with SYBR Green I and PI in a 1:100 ratio, followed by 15-minutes of incubation at 37 °C in the dark.

To ensure unobstructed functioning of the flow cytometer, an appropriate dilution of the semen samples was necessary prior to the analysis [27]. The native semen contained on average 300 million sperm cells/mL. For flow cytometric sperm analysis, the samples are usually diluted to concentrations of about 0.5 - 1 million sperm/mL. Due to decreasing sensitivity with lower numbers of bacteria, the aim was to find a dilution that meets the requirements of the flow cytometer while keeping TVC at detectable levels. In order to achieve this, we performed a 1:100 dilution (1:10 predilution at the station, followed by another 1:10 dilution with the mastermix) which led to an average sperm concentration of 3 million spermatozoa/mL and thus to an event rate of maximum 30,000 events per second, which should not be exceeded according to the manufacturer [28].

For the spiked semen samples, bacteria solution was added to the semen in a 1:10 ratio.

3.7 Flow cytometry

Analyses were performed using a CytoFlex (Beckman Coulter, Fullerton, CA, USA) equipped with a 488 nm laser (50 mW laser output). For detecting the green fluorescence a 525/40 nm bandpass filter was used and the red emissions were captured through a 610/20 nm band-pass

filter. Samples were analyzed at a speed of 0.5 µL/sec for 100 sec. Between samples a cleaning solution (FlowClean Cleaning Agent, Beckman Coulter, Fullerton, CA, USA) was run through the fluidics system of the cytometer for 10 sec, in order to prevent overspill of the following sample.

For compensation as well as to determine background fluorescence, an unstained sample and single stained control samples of unfiltered Evian® water were used. Stopping rule was set at 20,000 events in the bacteria gate and the compensation matrix was compiled automatically afterwards. To reduce the amounts of events conditioned by the ground fluorescence a threshold was used at the 525/40 nm bandpass filter.

Data analysis was done with the CytoFlex Software (Beckman Coulter, Fullerton, CA, USA). Gating was done as proposed by Hammes et al. [29] by using a green vs. red fluorescence intensity dot plot (Fig. 1). The bacteria enumeration (gate B2; plot C) was performed by analyzing samples of the seven bacteria species to set the region of interest. Thereafter, measurements of spiked semen samples followed in order to confirm that the region of appearance remained the same. Doublets were then excluded by using a forward scatter area (FSC-A) vs. forward scatter height (FSC-H) dotplot (plot D). For further control of the measurements, green fluorescence vs. time was plotted (plot E).

3.8 Bacterial count via MPN method

For comparative measurements, 1 mL of raw semen was sampled from each of 113 ejaculates and cultured in 9 mL tryptic soy broth (Thermo Fisher Diagnostics AG, Pratteln, Switzerland). Ten-fold dilution series in triplicates were prepared and the concentrations of viable microorganisms were estimated using the MPN method. The TVC was then enumerated after 48h culturing at 37 °C at aerobic conditions. To verify which bacteria were commonly found in the semen, the dilution 10^{-2} was streaked on Columbia blood agar (Thermo Fisher Diagnostics AG, Pratteln, Switzerland) and the isolates were identified by standard bacteriological procedures [30].

3.9 Data analysis

For data analysis the SPSS-Software was used (IBM® SPSS® Statistics, Version 23). To summarize the distribution of bacterial counts of these two methods, the median and median absolute deviation (MAD) were calculated. As the data were not normally distributed, the Spearman's rho correlation coefficient was used for analyzing the relation between measured and calculated bacteria counts of spiking experiments as well as the relation between bacterial counts determined by flow cytometry and the MPN method. For the assessment of the correlation between the measured and expected counts of the dilution series samples, the Kendall's tau coefficient was computed due to the small sample size. Statistical significance was set at $P < 0.05$ for correlation analysis.

To further evaluate the agreement between the MPN method and the flow cytometric assay (FC), the approach suggested by Bland and Altman [31] was used. Briefly, MPN and FC bacterial counts were log transformed as follows: $Z={}^{10}log(X+10)$, where Z is the logarithm to base 10 and X the original value, in order to achieve a lognormal distribution of the difference $d = MPN - FC$. Thereafter, the differences between paired measurements were plotted against the means of paired measurements to construct a Bland and Altman plot [31]. The lower and upper limits of agreement (LoA) for the log-transformed data were calculated as $\bar{d}$–1.96SD and $\bar{d}$ + 1.96SD, respectively; 95% confidence intervals (95% CI) were computed for the estimates of $\bar{d}$ and LoA of the log-transformed data. The concordance correlation coefficient (r_c) suggested by Lin [32] was calculated to evaluate the agreement of the two methods; r_c values <0.90 were considered to represent a poor agreement.

4. Results:

4.1 Determination of flow cytometric setting parameters

4.1.1 Pure cultures

Bivariate dot plots of red vs green fluorescence intensity were chosen to discriminate between viable and dead cells. *Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa* populations appeared as elongated clouds of events (Fig. 2B), whilst *Aeromonas* spp., *Escherichia coli, Staphylococcus aureus* and *Streptococcus* spp. were packed more closely (Fig. 2C). Dot plots of the bacteria mix showed a combination of these patterns (Fig. 2D).

Events scattered around the main cloud were only spread around the top left quarter and were denoted as dead cells, since they had a higher red and lower green fluorescence.

4.1.2 Spiking

When analyzing a semen sample four different clouds were identified in the dot plot (Fig. 3B). Due to their size, sperm cells have higher fluorescence intensity and appeared in the upper right corner (Fig. 3B, gate S). Live bacteria were gated in the region previously identified through analysis of pure cultures (Fig. 3B, gate B). The region marked D contained dead bacteria as well as debris. By gating these events back into a FSC/SSC-dotplot the difference in size becomes visible (Fig. 3C and3D). The ungated cloud in between was characterized as background noise from the semen extender, since it appeared in the same region as when analyzing extender samples (Fig. 3A).

The majority of semen samples showed low TVC numbers leading to only few events in region B (Fig. 4A, gate B). After adding bacteria to the samples events appeared on the flow cytometric dot plots in the expected region with the same pattern seen during the analysis of the pure culture samples (Fig. 4B).

4.1.3 Descriptive statistics of first experiment

The median of measured TVC was $7.6x10^7$ bacteria/mL with a minimum of $4x10^6$ bacteria/mL and a maximum of $2.4x10^8$ bacteria/mL. The median of MPN-predicted TVC values was $7.2x10^7$ bacteria/mL with a minimum of $4x10^6$ bacteria/mL and a maximum of $2.2x10^8$ bacteria/mL. TVC of the spiked samples strongly correlated ($r = 0.96$; $P < 0.001$) with the predicted amount (Fig. 5).

For the dilution series a strong correlation was found across all dilutions ($\tau=0.76$, $P<0.01$).

4.2 Comparative measurements with the MPN method

The median TVC (± MAD) of the 113 fresh semen samples analyzed by flow cytometry was $2.4x10^4 \pm 1.6x10^4$ bacteria/mL with a minimum of 0 and a maximum of $4.5x10^5$ bacteria/mL.

The median count of the samples analyzed with the MPN method was $2.4x10^4 \pm 2.2x10^4$ bacteria/mL. The minimum concentration was $2.4x10^2$ bacteria/mL and the maximum concentration was $1.1x10^6$ bacteria/mL. The correlation between the bacterial number determined by both methods was moderate ($r = 0.28$; $P < 0.01$; Fig. 6).

The mean difference $\bar{d}$ between the log-transformed bacterial counts assessed with MPN and FC, the estimated LoA as well as the respective 95% CI are shown in Table 1. The above mentioned statistical parameters and the regression line describing the relation between the two methods are graphically presented in a Bland and Altman plot (Fig. 7). As shown in Figure 7, the mean difference $\bar{d}$ of the log-transformed values was close to zero ($\bar{d} = -0.041$; Table 1); this implies that the mean ratio of the two methods $MPN/_{FC}$ approximated 1[1]. In an attempt to better conceptualize the results of the Bland and Altman analysis, the lower and upper LoA were back-transformed to the original scale [33]. Based on the anti-log values of the LoA, it appeared that the $MPN/_{FC}$ ratio can considerably deviate from 1 and vary between ~0.006 and ~129. As demonstrated in Figures 7 and 8, the comparison of the two methods revealed a proportional

[1]In particular, *(MPN+10)/(FC+10) = 0.910*

bias. In particular, it appeared that the difference between the two methods initially narrowed but further increased with increasing bacterial counts. The value of Lin's concordance coefficient was $r_c = 0.27 < 0.90$ implying a poor agreement between the two methods.

5. Discussion:

In the present study we developed a method to count TVC in fresh boar semen by flow cytometry using a live/dead-staining. In particular, we chose SYBR Green I, an unspecific cell permeable DNA-dye, and Propidium Iodide (PI), a DNA-dye which penetrates only the membrane of dead cells. Both dyes can be excited by the light of a blue laser included in nearly all basic flow cytometers, which gives the opportunity to use the staining not only in well-equipped research centers but also under field conditions [34]. SYBR Green I is frequently chosen for bacterial staining in various media, including water [18,34,35,36] and soil [25] and PI is a common counterstain to enable detection of dead cells [36,37]. The combination of SYBR Green I and PI was also approved in several studies to be suitable for discrimination of viable and dead environmental bacteria [34,38,39,40]. There are other options available like a double staining with SYTO9 and PI [41,42] or DAPI (4,6-diamidino-2-phenylindole) and PI [43]. Nevertheless SYBR Green I remains the most widely used DNA-dye for counting bacteria in aquatic and soil samples [40,44]. In preliminary tests we could achieve the best separation of bacteria from background noise and debris using the combined SYBR Green I and PI staining (data not shown).

Although PI is a very common nuclear- and chromosome counterstain [37] to mark dead cells, it is suspected to also stain some bacteria during a short period of their life cycle [45]. During the bacterial growth phase PI seems to be able to penetrate the cell wall; the reason for this though is still unclear [45]. Either entry of the dye through shortly open cell wall structures or by the divisome escorted cell division process is suspected to be the cause, but further research is needed to identify the mechanisms [46]. However this effect appears to be strongly dependent

on bacteria species, as for example the tested *Mycobacterium* strain was highly susceptible to take up PI during the growth phase (45% of the PI stained cells were still culturable) whereas *E. coli* (4% culturable) was not [45]. Taking into account the complex composition of the bacterial variety in boar semen, the effect of faultily marked as non viable bacteria could be higher in some samples than others and, thus, be one reason for the moderate correlation of counts acquired via MPN method and flow cytometry. Though little is still known about this effect, apart from *E. coli* none of the used bacteria species are further investigated in respect of this effect, and PI remains the favored dye to mark dead cells in bacteria populations [19,46].

As reference method we decided to use the MPN-method, which resulted in rather grouped bacterial counts pattern in comparison to the TVC values compiled by flow cytometry (Figg. 6 and 8). According to the Bland-Altman-analysis, the difference between the two methods is fortified and the result is more prone to being over- or underestimated for ejaculates with very low and very high bacterial counts, respectively (Fig. 8).

In order to further investigate whether the flow cytometric measurements are working reliably at different concentrations of bacteria we did dilution series with spiked semen samples, starting off with a bacteria concentration of 10^7 bacteria/mL down to a concentration of about $4.5x10^5$ bacteria/mL. According to several different studies [4] boar semen usually contains about 10^4 - 10^6 cfu/mL, which was the basis for choosing these concentrations as these measurements were done prior to the comparison analysis. The main target of the developed method should be the identification of highly contaminated samples, which could forfeit quality due to their bacterial abundance. Although there is no general cutoff value at which concentration TVC is detrimental for boar sperm, there are a few studies on the dose dependency of single bacteria species reporting concentrations of 10^7 - 10^8 cfu/mL as problematic for sperm quality [7,47,48]. Nevertheless further studies should be done in order to identify the source of the bias between the FC and MPN method employed in our study.

As determining the TVC in semen is not a daily routine procedure in bacteriology, no fixed standard method and hardly any literature can be found to resort to in order to choose the best

reference method. In most studies plating techniques have been used [9,49]; nevertheless, in these studies identifying the TVC as precisely as possible was not the matter of particular interest. Especially in the field of drinking water analysis, many microbiological studies have been carried out in the past, in an attempt to establish new methods for the evaluation of bacterial counts. Flow cytometry is one of them and has been vastly used in drinking water research for more than a decade now, leading to a considerable amount of data from multiple full-scale studies [19]. In addition to many studies reporting poor correlations of plate count techniques and flow cytometric measurements [50,51,52,53], a retrospective analysis done by van Nevel et al., compiling more than 1800 data points, "shows extremely weak correlation" [19]. The staining used in the according studies has also been based on SYBR Green I and PI like in our experiments. A broad number of analytical techniques is nowadays available for bacterial enumeration with a shift from classical culture techniques towards molecular technologies like immunoassays, PCR and the detection of biomolecules, like adenosine triphosphate (ATP) [54]. Interestingly, the latter has been shown to strongly correlate with TVC attained by flow cytometry [19]. Taking this into account the moderate correlation between the TVC acquired by MPN and flow cytometry in the present study is not surprising. However, further studies with comparison to other microbiological techniques are necessary to securely determine the source of the observed moderate correlation and discrepancy in the Bland-Altman-plot.

One important step when working with flow cytometry is to ensure that the cells are neither agglutinated nor attached to other particles, which can lead to underestimation of the actual count [25]. It is a well-known problem that more vigorous methods like blending or sonication can lead to damaged cells altering the result of the flow cytometric measurement [55,56]. Flow cytometric assays for assessing bacterial counts in milk samples commonly include protease enzymes to extract protein globules to avoid interferences from the milk matrix [22,23,57], while others stated a detrimental effect of these enzymes on certain species of bacteria [56]. Preliminary experiments in our lab including sonication, filtering, and the application of

enzymes led to an increased portion of debris and a less distinct segregation of the bacteria cloud in the dot plots of the flow cytometric analyses. For this protocol we chose to use a vortex to mix and separate bacteria from particles and sperm and solve agglutinated bacteria clusters. Nonetheless some bacteria are more prone to form clumps and chains than others and, depending on the bacterial composition of the native ejaculate, this can lead to a slight underestimation of the bacterial count [23,58]. The more bacteria are included in the ejaculate the more they tend to cluster which could be one reason for the bias between the results obtained by the MPN method and flow cytometry in samples with high numbers of bacteria. Whether flow cytometry gives an underestimation or the MPN method an overestimation of the actual TVC would need to be further investigated as discussed above.

The different bacteria species showed diverse dot plot patterns. Most were distributed in a more longish pattern (Fig. 2B) whilst especially *Staphylococcus aureus* and *Streptococcus* spp. appeared in a more confined cloud (Fig. 2C). The reason for this is most likely the different shape of the involved bacteria species. *Klebsiella pneumoniae, Aeromonas* spp., *Pseudomonas aeruginosa, Escherichia coli* and *Proteus mirabilis* are all rod-shaped species, which leads to various possible measurement angles and thus to more widespread dotplots. On the other hand, coccoidal species display a more centered cloud due to their round and globular shape [25].

The composition of the bacteria might also have an influence on the estimated TVC due to changes in the population during the culturing time of 48 hours. Some bacteria strains, for example *Pseudomonas aeruginosa,* are known to produce bacteriocins, that act as growth inhibitors for other bacteria species in mixed cultures [59,60]. Additionally, other proteins are also known to be involved in bacterial interactions [61]. Not only growth inhibition but also induced cell lysis can be mediated by specific enzymes [62]. These interactions of bacteria influencing growth dynamics may lead to a different bacterial count assessed after 48h of culturing in contrast to the immediate enumeration via flow cytometer.

With the presented method we are able to determine the TVC of a fresh boar semen sample in order to monitor its microbiological quality. Flow cytometric protocols for the differentiation of

gram positive and negative bacteria in milk have been developed [21,22], but still more studies are needed to have more distinguished information about the bacterial compounds of semen samples.

6. Conclusion:

This study demonstrates an alternative method to assess the TVC in boar semen, focusing on the detection of highly contaminated ejaculates. The presented flow cytometric protocol makes it possible to distinguish between viable and dead bacteria in fresh semen samples without the need of multiple processing steps before measurement. In contrast to classical microbiological plate count techniques, it is less time and labour consuming and, thus, enables an on-line evaluation of produced semen batches.

7. Acknowledgements:

We thank Natasha Carroli for the technical work regarding all microbiological procedures and Roger Stephan for proofreading the manuscript. In addition we would like to thank SUISAG, Sempach, Switzerland, for financial support and especially all staff members of the AI stations for their support on collection of the semen samples.

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9. Figures

Figure 1: Semen sample with added bacteria. Due to debris and other small particles originating from the semen extender and the ejaculate separation of the bacteria population (B1) in the forward scatter area (FSC-A) vs. side scatter area (SSC-A) density plot was not distinct (plot A, plot B(zoom)), so a green (FITC-A) vs. red (ECD-A) fluorescence intensity dot plot was used for the bacteria enumeration gate B2 (plot C) as established by Hammes et. al. [29]. Spermatozoa are marked with S (plot A) and S2 respectively (plot C). For defining that gate samples of different bacteria species were measured first separately and then combined with semen samples to define the region of interest. Doublets were excluded (B3) by a dotplot of forward scatter area (FSC-A) vs. forward scatter height (FSC-H) (plot D) and FITC-A vs. time was additionally plotted to further ensure a reliable and unobstructed measurement (plot E).

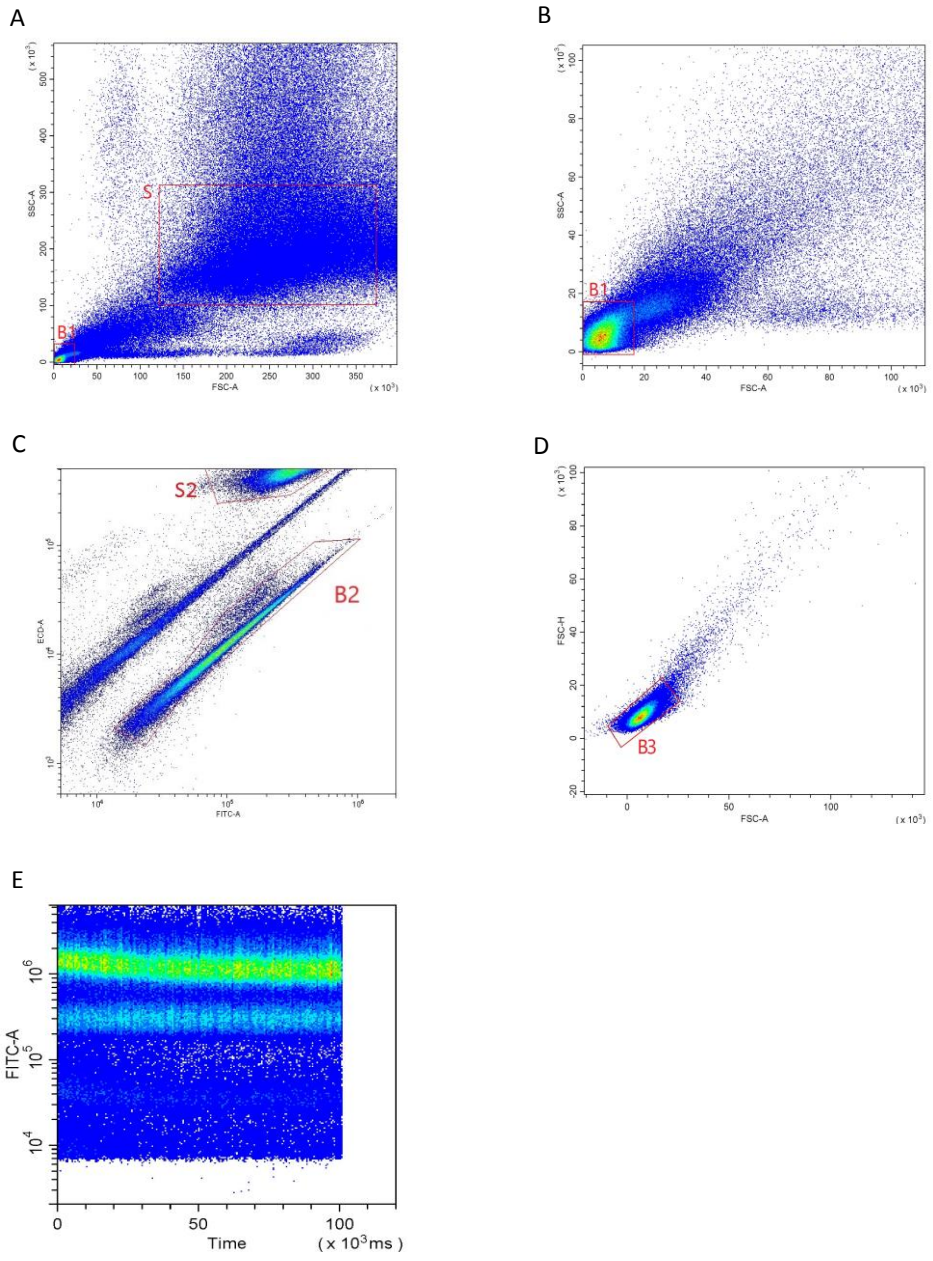


Figure 2: Analysis of pure cultures of bacteria with the viable-dead staining using a density dotplot of green (x-axis) and red (y-axis) fluorescence intensity. To ensure that there is no contamination in the staining solution an aliquot was incubated at 37 °C for 15 min in the dark and then measured first as a negative control (A). Due to differences in shape and size some species like *Proteus mirabilis* appeared as an elongated cloud (B) while others exhibited more compact distribution (C). The dot plot of the mixed sample of all bacteria species reflected the before assembled single species patterns (D). Events outside the gate B are dead bacteria cell as well as debris.

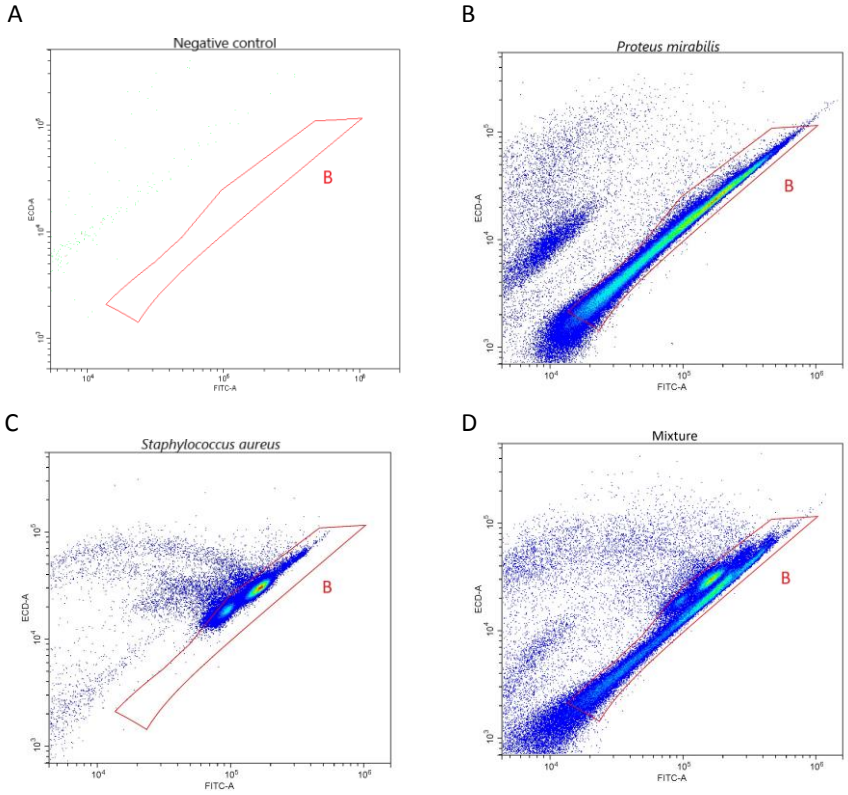


Figure 3: Figure Density dotplots of green (x-axis) vs. red (y-axis) fluorescence intensity. Figure A shows the analysis of the semen extender which was measured as a negative control sample to ensure that no bacterial contamination originated either from the semen extender nor the staining solution of SYBR Green I and PI. The ungated beam is considered to be background noise caused by the extender and can also be recovered in analysis of the semen sample (Fig. B). Sperm cells are located in gate S, while the considerably smaller viable bacteria appeared in gate B, the same region as before identified by analyzing the pure culture samples (Fig. 2). Debris and dead bacteria are merged in region D.

When backgating the debris in gate D (Fig. C) into a FSC/SSC- dotplot the difference in size in contrast to sperm cells (Fig. D, gate S gated back) can be seen.

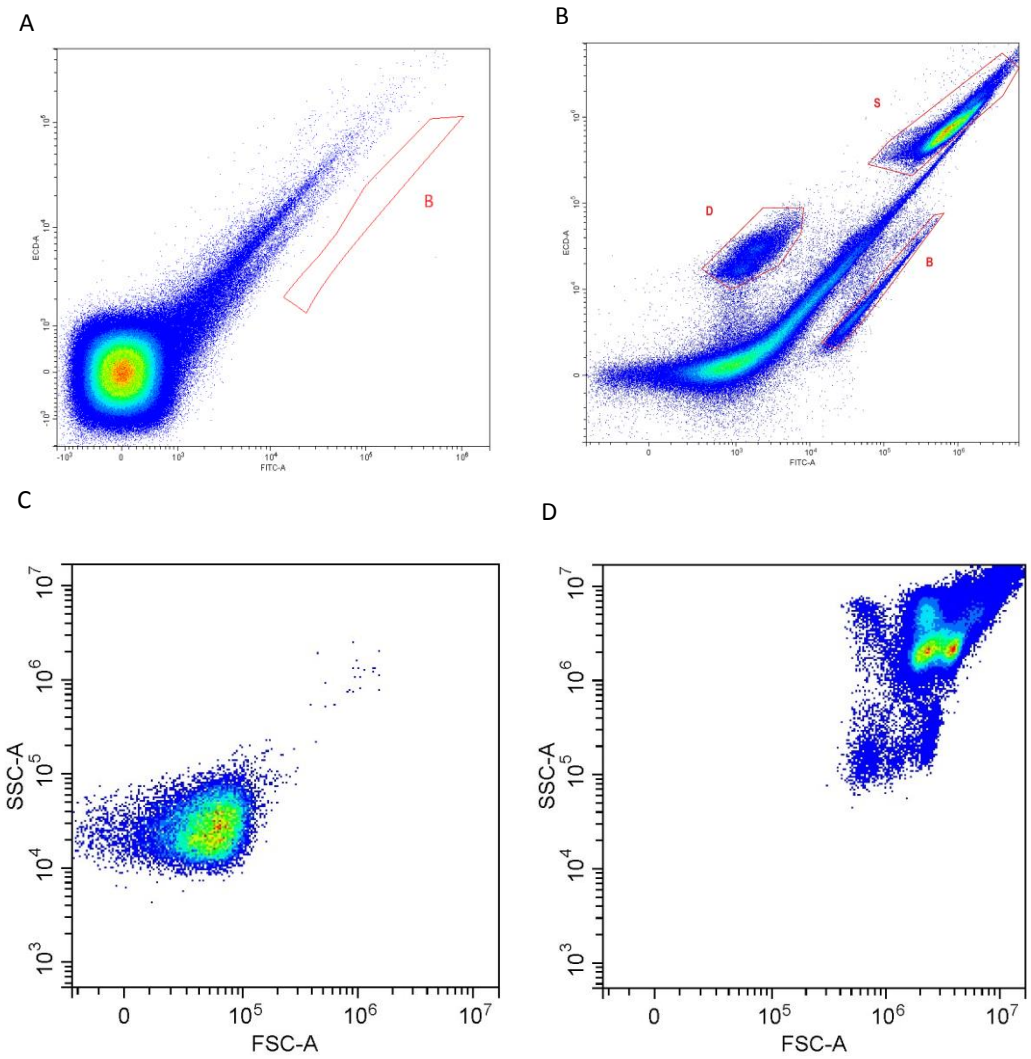


Figure 4: Analysis of a semen sample before (A) and after (B) spiking with bacteria. The bacteria appeared in the same region (gate B) as before when analyzing solitarily the bacteria in pure cultures.

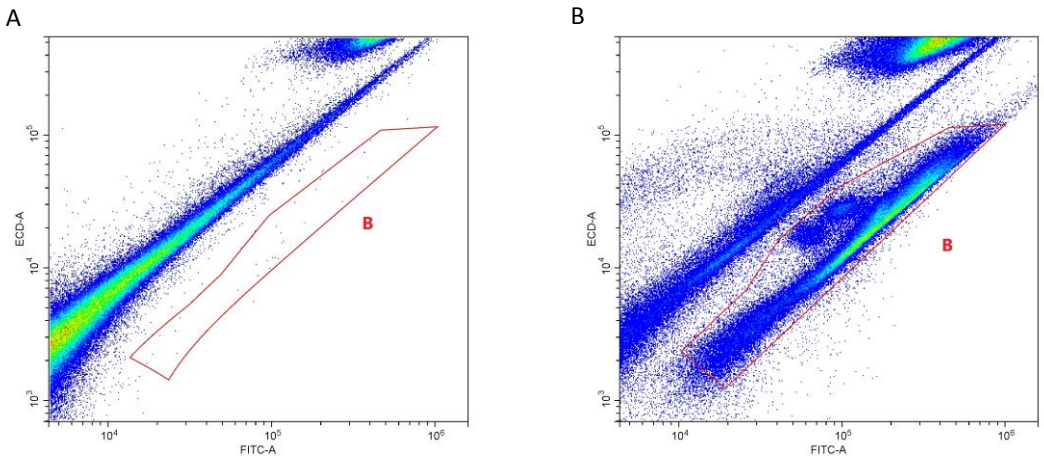


Figure 5: Scatter plot of the strong correlation of expected and calculated TVC (r=0.96; P < 0.001).

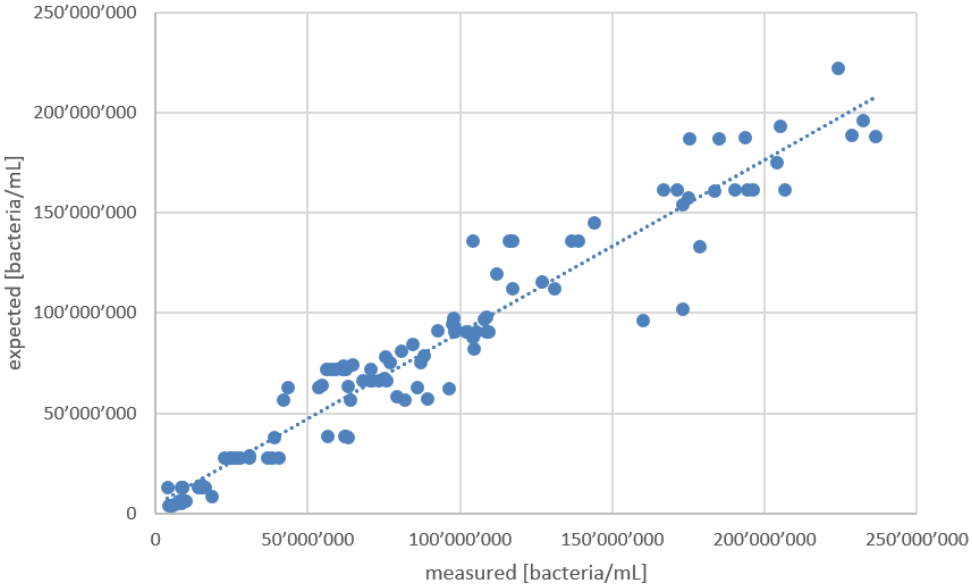


Figure 6: Scatter plot of the moderate correlation of TVC determined by flow cytometry and the MPN method (r=0.28; P < 0.01).

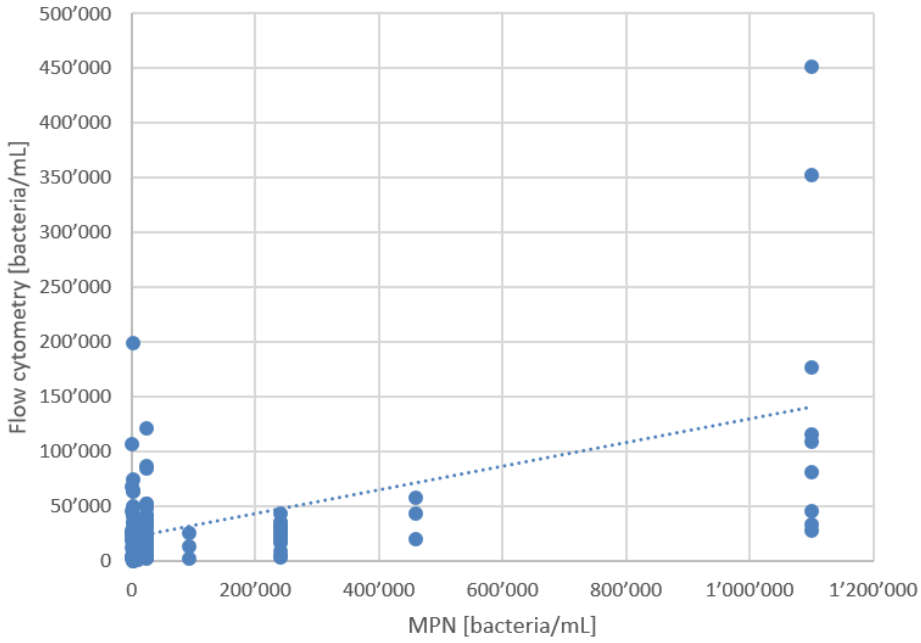


Table 1: Bland and Altman plot statistics, including the estimated mean difference between the log-transformed bacterial counts determined by the MPN-technique and flow cytometry (n=113), the estimated upper and lower limits of agreement. The 95% CI of estimates were also calculated.

	Estimate	**Lower 95% CI**	**Upper 95% CI**
Mean difference ($\bar{d}$)	–0.041	–0.246	0.164
Lower limit of agreement	–2.195	–2.546	–1.844
Upper limit of agreement	2.113	1.762	2.464

Figure 7: Bland and Altman plot for the bacterial counts (log-transformed) assessed by the Most Probable Number method and flow cytometry; the differences between the two methods are plotted against their means. From bottom to top, the horizontal dot-dashed lines represent the estimated lower limit of agreement, the mean difference ($\bar{d}$) between the two methods and the upper limit of agreement; the respective dashed lines represent the 95% CI of the above mentioned estimates (the green, blue and orange shaded areas, respectively). The regression line (blue line) describes the relation between the differences and the means with 95% CI (grey shaded area).

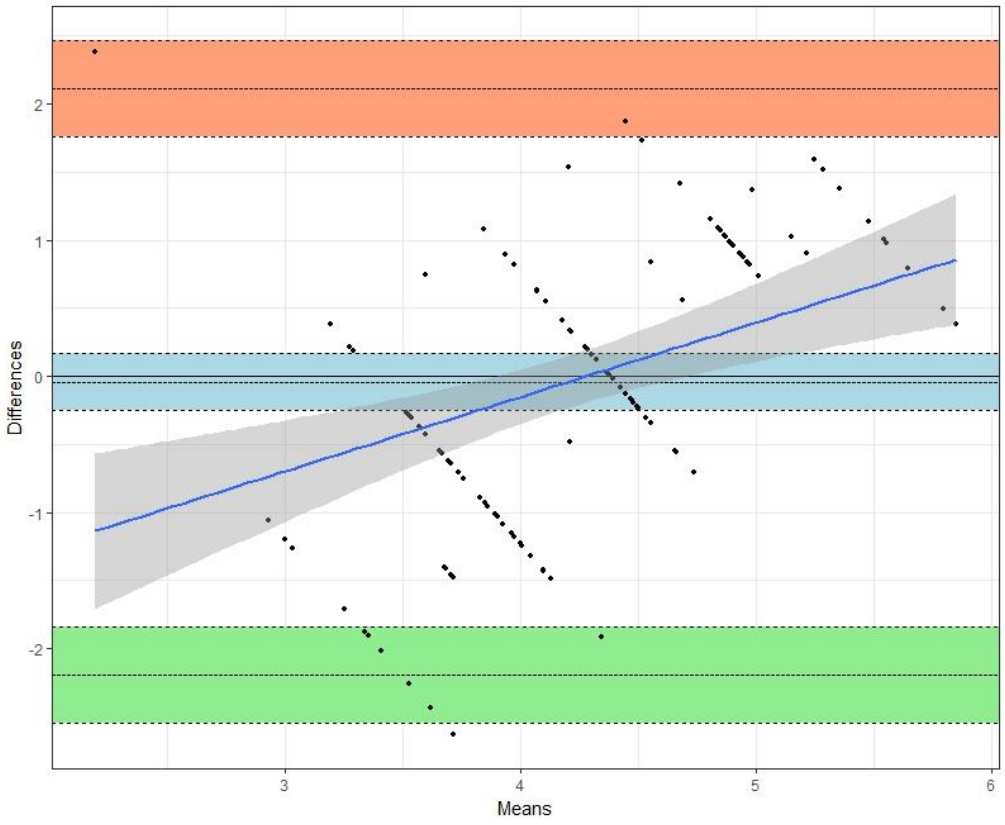
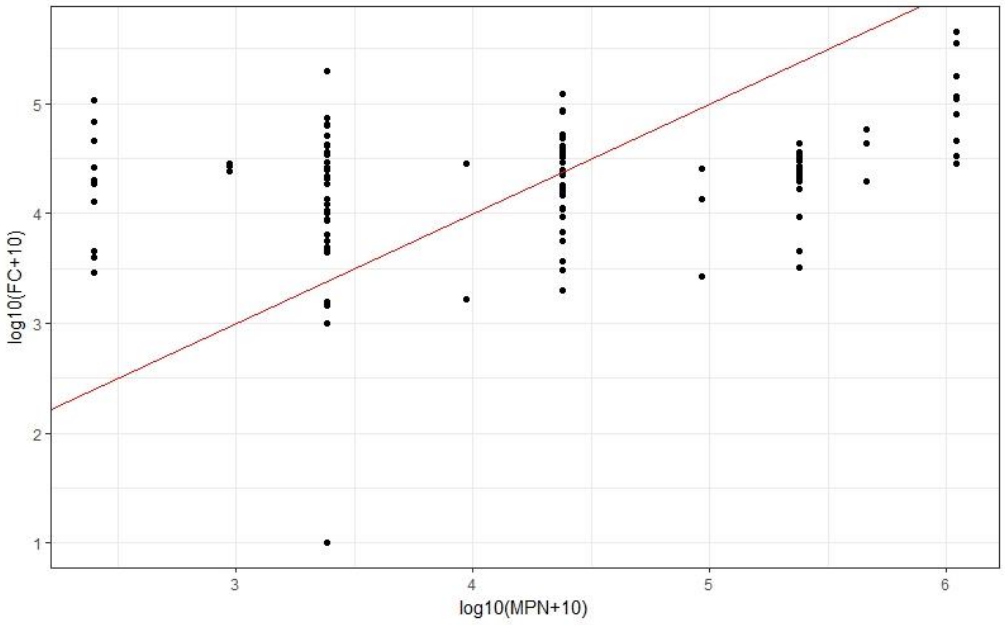


Figure 8: Dot plot of the log-transformed bacterial counts assessed using the Most Probable Number method (MPN) and flow cytometry (FC). The red line represents the line of equality (x =y). For low numbers of bacteria, FC gave an overestimation of bacterial count in comparison to the MPN method; however, bacterial counts were underestimated when samples with high number of bacteria were flow cytometrically examined.



Danksagung

Die Entstehung dieser Arbeit wäre ohne die tolle Unterstützung aller Beteiligten nicht möglich gewesen.

Als erstes möchte ich meinem Betreuer und Doktorvater Fredi Janett ganz herzlich für all die geleistete Arbeit danken. Sei es bei der Versuchsplanung und Materialbeschaffung, beim Einfrieren von Pailletten zu später Stunde während vieler Versuchswochen oder aber durch wertvollen fachlichen Input sowie mentale Unterstützung bis zur Veröffentlichung des Papers – stets habe ich ein offenes Ohr gefunden und Hilfe in allen Belangen. Mein herzlichstes Dankeschön dafür!

Mein weiterer Dank gilt Heiner Bollwein, insbesondere für seine fachliche Unterstützung im Bereich der Durchflusszytometrie. Trotz zahlreicher Hürden von den ersten Versuchen bis zur Veröffentlichung des Papers hat er die Hoffnung nie aufgegeben und mich stetig bei der Entwicklung unterstützt, wofür ich ganz herzlich danke!

Ein besonderer Dank gilt Sarah Wyck, die mir nicht nur unermüdlich beim Beschriften hunderter Pailletten geholfen hat, sondern auch sonst stets zu spontanen Aktionen aller Art bereit war, allen voran die legendären Dienstagabende. Aus guter Kollegialität hat sich eine feste Freundschaft zum Pony stehlen entwickelt, die ich nicht mehr missen möchte.

Ein grosses Dankeschön gebührt zudem Eleni Malama, die mit unerschöpflicher Geduld auch die trivialsten Statistikfragen beantwortet und mit ihren Analysen und Anmerkungen einen grossen Teil zum Gelingen der Arbeit beigetragen hat. Egal ob früh oder spät, Wochenende, Ferien oder Mutterschutz, Eleni hatte stets ein offenes Ohr für Anliegen jeglicher Art, und doch blieb beim grössten Stress immer noch Zeit für einen Fondueabend oder vorweihnachtliches Kekse backen, was ohne Nikos, Emmeleia und Nefeli auch nur halb so lustig gewesen wären.

Auch allen anderen aus meinem Team vom alten Strickhof möchte ich ganz herzlich für die Unterstützung danken, insbesondere Pia Weissbeck, Andreas Fleisch, Firat Korkmaz, Ozancan Arslan, Sarun Keo und Hanspeter Müller.

Die gesamten Versuche wären jedoch ohne die tolle Unterstützung der Teams der SUISAG KB-Stationen in Knutwil und Wängi nicht möglich gewesen. Allen voran möchte ich Ruedi Gugelmann danken, der mir das Vertrauen geschenkt hat, das Projekt und die Aufgaben als Stationstierärztin zu übernehmen und mich stets dabei unterstützt hat, wo immer es ging. Auch Benno Hodel danke ich ganz herzlich, dass bei den vielen Herausforderungen der Absamungsplanung meine Wünsche auch immer noch berücksichtigt wurden. Abschliessend gilt mein besonderer Dank noch den beiden Stallteams in Knutwil und Wängi sowie dem Laborteam in Knutwil, die mir stets dabei geholfen haben, dass ich die Proben möglichst optimal gewinnen konnte.

Last but noch least möchte ich ganz besonders meiner Familie danken, ohne deren grosser Unterstützung das alles nicht möglich gewesen wäre. Ein spezielles Dankeschön gilt hier meinem Mann Jens, der nicht nur während der intensiven Versuchsphase viel zurück stecken musste und mich bei Tiefschlägen immer wieder aufgebaut und stets an mich geglaubt hat, als auch meinen Eltern, die es mir trotz eines weinenden Auges ermöglicht und mich ermutigt haben, den Schritt in die Schweiz zu wagen. Ihr seid einfach grossartig!

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